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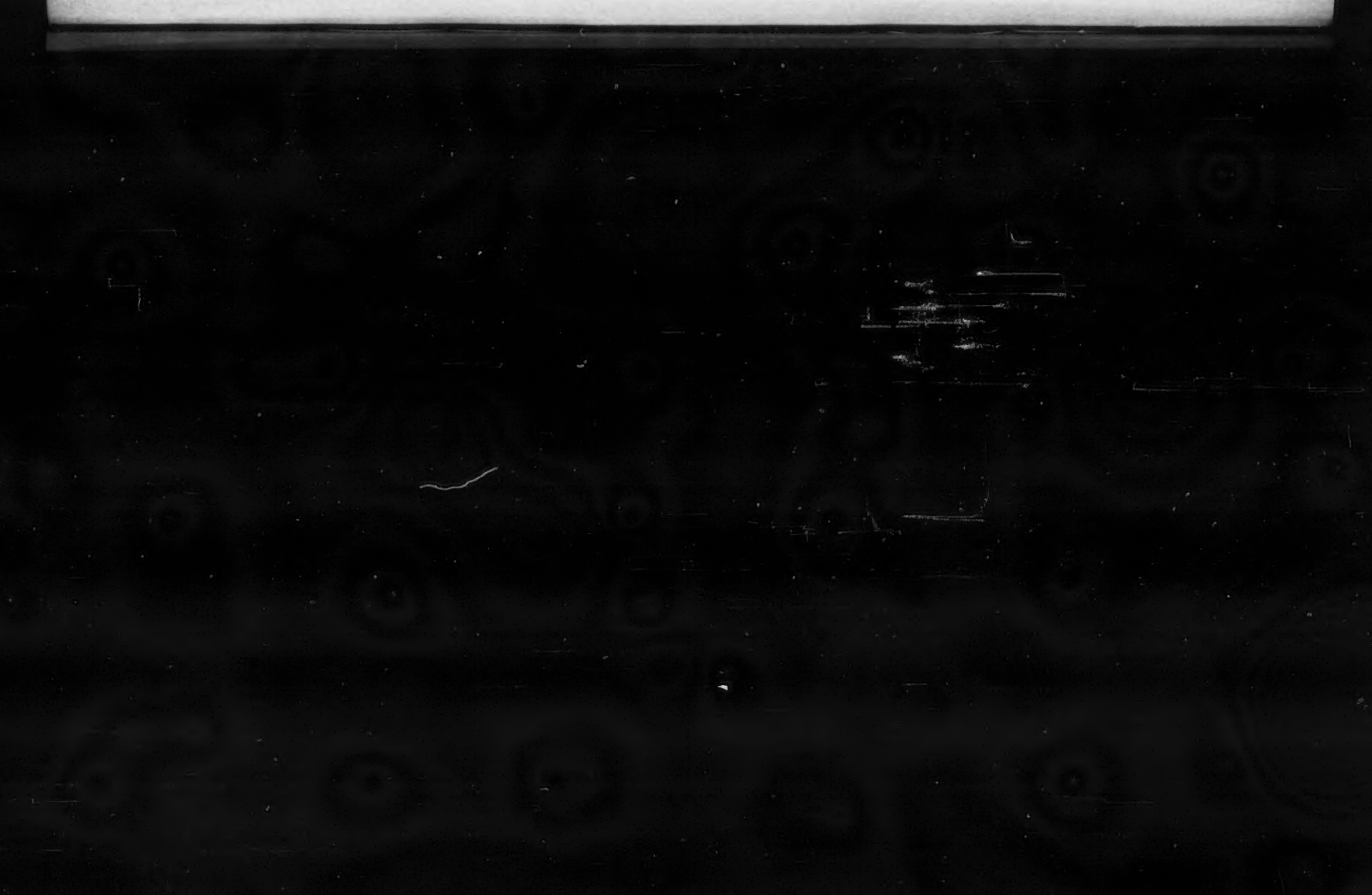

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FERMENTATION OF BEET MOLASSES BY *PSEUDOMONAS HYDROPHILA*[1]

BY D. MURPHY,[2] R. W. WATSON,[2]
D. R. MUIRHEAD,[3] AND J. L. BARNWELL[4]

Abstract

Strains of *Pseudomonas hydrophila*, isolated from freshwater fish, dissimilate beet molasses sugars aerobically to yield up to 96% of theoretical 2,3-butanediol + acetoin in 25 hr. at 32°C. Analyses of end-products for 11 fermentations of diluted Chatham 1946 molasses containing approximately 5% total sugar show that strain 492 converts on the average 98.1% of the sugar in 25.5 hr. without pH control. Yields average 87.8% of theoretical diol + acetoin, 13.1% ethanol, and 2.2% organic acids. The highest-yielding organism previously known, *Aerobacter aerogenes* B199, on the same substrate, under the same conditions, averages 82.9% theoretical diol + acetoin, 19.8% ethanol, and 4.5% organic acids in 24.5 hr. Aeration has less tendency to convert diol to acetoin in the *P. hydrophila* fermentation. It therefore appears that *P. hydrophila* 492 produces a lighter yield of glycol + acetoin than *A. aerogenes* B199. Under certain conditions, *P. hydrophila* produces approximately 95% of the theoretical glycol, and no ethanol. Slight variations in fermentation time and product yield occur in molasses media from different sources. Fort Garry and Alberta molasses (Steffen and non-Steffen) provide average yields of 88.9% theoretical diol + acetoin in 25 hr. The mixture of stereoisomers from *P. hydrophila* 491 comprises 50% *racemic*, 48% *meso-*, and 2% *levo-* 2,3-butanediol.

Introduction

Lemoigne first established acetylmethylcarbinol and 2,3-butanediol production by species of *Proteus* (2, 5). The report of a positive Voges–Proskauer reaction in pseudomonads by von Wolzogen Kühr (4) led Stanier and Adams to investigate the nature of the *Aeromonas* (*Pseudomonas*) *hydrophila* fermentation (16). Examination of strains from "red-leg" diseased frogs revealed that *Pseudomonas hydrophila* carries out a typical 2,3-butanediol fermentation similar to that of *Aerobacter aerogenes* (15, 16). No further work of a biochemical nature on *P. hydrophila* has hitherto been reported.

In the present investigation yields of 2,3-butanediol + acetoin up to 96% of theoretical have been obtained using Reed's strains of *P. hydrophila*. These organisms, isolated from pike and salmon (11, 12), differ from those employed

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by Stanier and Adams, in that they produce a diol containing a high percentage of the *racemic* mixture, give higher yields of glycol, and ferment sugars more rapidly and completely (1). In vigor and stability these strains resemble the best strains of *A. aerogenes*. Under the conditions described in this communication they give less ethanol and acetoin, and higher yields of 2,3-butanediol than *A. aerogenes* B199.

Experimental

P. hydrophila strains R1 (N.R.C. 491) and R2 (N.R.C. 492), isolated from salmon and pike respectively, were obtained from Dr. G. B. Reed of Queen's University, Kingston, Ont. *A. aerogenes* M 148 was isolated locally from soil. *A. aerogenes* B199 was recently obtained from the Northern Regional Research Laboratory, Peoria, Ill.

The basal media, of beet molasses* diluted 10-, 6.7- or 5-fold with tap water, contained approximately 5%, 7.5%, and 10% sucrose respectively. Samples of molasses were stored at 0°C. Prior to dilution each sample was left overnight at room temperature, and thoroughly mixed by prolonged agitation. Dibasic ammonium phosphate (0.10% in a medium containing 5% sugar, 0.15% in one containing 7.5% sugar, and 0.20% in 10% sugar), along with equal amounts of ground bran (14) were added, and the pH of the medium adjusted to 5.6 with acetic acid before sterilization. Two hundred and fifty milliliters of medium was sterilized in each of the special flasks used in pH-controlled fermentations (8). Leeds and Northrup electrodes, designed for sterilization by ultraviolet light (19), and used with long shielded leads, allowed the pH of the medium to be determined without opening the flasks. To prevent foaming, 5 to 10 drops of sterile corn oil was added to each flask prior to inoculation. Addition of 4% inoculum brought the final volume to 260 ml. All fermentations were carried out under an air flow of about 15 ml. per min. in a shaker cabinet (8) with the temperature controlled at about 32°C.

Inocula were prepared by transferring organisms from a 48 hr. agar slant to 50 ml. of sterile stock solution, made by 10-fold dilution with tap water of the molasses under investigation. Incubation in an Erlenmeyer flask on a shaker at 32°C. for 18 to 22 hr. gave an inoculum that provided vigorous growth in all molasses samples investigated. At the end of each fermentation the purity of the culture was tested by plating, and by the examination of Gram-stained smears.

Methods of analysis for 2,3-butanediol, acetoin, ethanol, and carbon dioxide have been described (7). Total organic acids were determined by titration, and estimated as acetic acid. Invert sugar in molasses samples was estimated after hydrolysis by the Underkofler semimicro modification of the Shaffer–Somogyi method, standardized with hydrolyzed sucrose (17). Molasses

* *Chatham molasses was obtained from the Canada and Dominion Sugar Co. Limited, Chatham, Ont. Samples of beet molasses were also provided by the Manitoba Sugar Co. Ltd., Fort Garry, Man., and the Canadian Sugar Factories Limited, of Raymond, Alta.*

sucrose was also determined by the double acid polarimetric method (9). A new method for the rapid estimation of stereoisomeric 2,3-butanediols will be described in a subsequent communication (20).

Results

Analyses of end-products for 11 aerobic dissimilations of diluted Chatham 1946 molasses by strain 492 are given in Table I. Conditions in these fermentations were nearly identical, except for variations in the initial pH, adjusted after sterilization by addition of sterile 2*N* phosphoric acid. Ethanol yields vary from 0 to 40 (average 26.2) mM. per 100 mM. of invert sugar fermented. Production of total diol + acetoin varies from 76.5% to 96% of theoretical, with an average of 87.8 mM. per 100 mM. of invert sugar fermented. The average fermentation time was 25 hr., during which 98.1% of the available sugar was dissimilated. In two fermentations, no ethanol, and approximately 95% of the theoretical diol was formed. High carbon recoveries are attributable to addition of acetic acid in the presterilization adjustment of pH (6). Measurement of pH during the fermentation shows that at no time did it fall below 5.0.

Effects of variation in the composition of molasses from a single factory over a period of three years are shown in Table II. Each sample of Chatham molasses was diluted 10-fold with water to contain 4.97%, 5.43%, and 5.51% of total sugar respectively (9, 17). The percentages of end-products from the 1947 and 1949 samples are strikingly similar. In the three fermentations the available sugars were 99% fermented in an average time of 21.3 hr. to yield 82.6% of theoretical diol + acetoin.

Analyses for aerobic dissimilations of diluted Fort Garry and Alberta (Steffen and non-Steffen) molasses containing 4.4%, 4.9%, and 5.0% initial sugar respectively are reported in Table III. An average of 98.5% of the sugar was fermented in 25 hr. to yield 88.9% (average) diol + acetoin, and 18.8% ethanol. Diol–ethanol ratios vary from 3.6 to 5.5 (average 4.7).

Effects of molasses concentration on diol yields using unacclimatized organisms were determined by varying the dilution factors for Chatham 1946 molasses. Results with approximately 5.0%, 7.5%, and 10.0% sugar in the medium are given in Table IV. With an initial sugar concentration of 10%, strain 492 utilized 70.7% of the sugar in 72 hr. A concentration of 7.5% in molasses was fermented in 24 hr. to yield 85.6% of theoretical diol + acetoin.

The fermentation of 10-fold diluted Chatham 1946 molasses by *A. aerogenes* B199 and N.R.C. strain M 148 was compared with the fermentation by *P. hydrophila* 492 on the same substrate under the same conditions (Tables I and V). Averaging the results in Table V shows that *A. aerogenes* B199 dissimilates aerobically 99.4% of the initial sugar in 24.5 hr. to an average yield of 82.9% diol + acetoin, 19.8% ethanol, and 4.5% organic acids,

TABLE I

AEROBIC DISSIMILATION OF 5% SUGAR IN CHATHAM 1946 MOLASSES BY *P. hydrophila* N.R.C. STRAIN 492

(mM. per 100 mM. invert sugar fermented)

Product \ Fermentation No.	1	2	3	4	5	6	7	8	9	10	11	Average
2,3-Butanediol + acetoin	95.0	96.1	88.2	91.7	87.9	88.7	85.3	88.4	87.3	76.5	81.4	87.8
2,3-Butanediol	95.0	95.4	82.4	76.2	75.0	84.7	81.3	77.7	76.2	71.3	77.5	81.2
Acetoin	0.0	0.7	5.8	15.5	12.9	4.0	4.0	10.7	11.1	5.2	3.9	6.6
Ethanol	0.0	0.0	39.9	23.7	29.3	26.4	27.9	27.1	34.1	39.8	40.0	26.2
Acids	6.5	5.7	....	4.9	0.0	6.7	5.3	5.3	2.3	3.2	3.9	4.4
CO_2	221	227	191	204	201	189	187	195	197	190	188	199
Diol–ethanol ratio	....	....	4.4	7.5	5.9	6.5	6.0	6.4	5.0	3.8	4.0	5.5
C recovery, %	102.3	103.8	103.9	104.7	101.9	101.7	99.1	102.2	103.2	97.0	100.2	101.8
Fermentation time, hr.	26	26	21	30	30	24	24	28	32	21	19	25.5
Sugar used, %	97.4	97.5	99.3	96.7	96.7	98.3	98.6	98.7	98.7	98.4	98.4	98.1
Initial pH 32°C.	6.18	6.18	5.65	5.40	5.42	5.60	5.80	5.40	5.03	5.42	5.85	
Final pH 32°C.	5.25	5.32	5.49	5.34	5.28	5.30	5.40	5.40	5.28	5.29	5.32	

estimated as acetic acid. Diol–ethanol ratios range from 3.6 to 4.9 (average 4.1). N.R.C. strain M 148 carries out a similar fermentation.

The percentage of each stereoisomer present in the glycol produced by *P. hydrophila* 491 was estimated by a physical method (20) and the results were checked by preparation of the di-*p*-nitrobenzoyl esters (13). Vacuum distillation of 25 ml. of crude *Pseudomonas* 491 diol, obtained by ether extraction of 1500 ml. of molasses liquor yielded an almost anhydrous stereoisomeric mixture with $n_D^{25} = 1.4339$ and $\alpha_D^{25} = -0.3$. From these two measurements it may be calculated that the isomeric mixture contained approximately 50% *racemic*, 48% *meso-*, and 2% *levo-* 2,3 butanediol (20).

The di-*p*-nitrobenzoyl esters of 1 gm. of purified *Pseudomonas* 491 diol were prepared by a modified Schotten–Baumann reaction (13) and recrystallized

TABLE II

FERMENTATION OF CHATHAM BEET MOLASSES FROM DIFFERENT CROP YEARS BY *P. hydrophila* STRAIN 492

(mM. per 100 mM. invert sugar fermented)

Product \ Crop year	1947	1948	1949
2,3-Butanediol + acetoin	85.1	77.2	85.6
2,3-Butanediol	79.4	70.7	79.8
Acetoin	5.7	6.5	5.8
Ethanol	28.7	28.4	31.3
Acids	8.0	5.4	5.3
CO_2	194	195	197
Diol–ethanol ratio	5.8	4.9	5.3
C recovery, %	101.3	96.3	102.1
Fermentation time, hr.	24	20	20
Sugar used, %	99.1	98.1	98.8
Initial pH (32°C.)	5.80	5.83	5.85
Final pH (32°C.)	5.66	5.30	5.50

TABLE III

FERMENTATION OF FORT GARRY AND ALBERTA BEET MOLASSES BY *P. hydrophila* STRAIN 492

(mM. per 100 mM. invert sugar fermented)

Product \ Source of molasses	Fort Garry	Alberta Steffen	Alberta non-Steffen
2,3-Butanediol + acetoin	93.1	84.1	89.6
2,3-Butanediol	90.0	80.7	83.8
Acetoin	3.1	3.4	5.8
Ethanol	35.9	45.4	31.7
Acids	2.5	4.8	1.4
CO_2	230	207	208
Diol–ethanol ratio	5.1	3.6	5.5
C recovery, %	108.2	106.1	104.1
Fermentation time, hr.	22	22	32
Sugar used, %	98.4	97.9	99.3
Initial pH (32°C.)	5.48	5.44	5.48
Final pH (32°C.)	6.15	5.68	5.61

TABLE IV

EFFECTS OF SUGAR CONCENTRATION ON AEROBIC DISSIMILATION OF MOLASSES SUCROSE BY *P. hydrophila* STRAIN 492

(mM. per 100 mM. invert sugar fermented)

Product \ Sugar concentration	5%	7.5%	10.0%
2,3-Butanediol + acetoin	88.2	85.6	90.5
2,3-Butanediol	82.4	84.8	81.0
Acetoin	5.8	0.8	9.5
Ethanol	39.9	36.9	20.5
Acids	....	0.5	12.8
CO_2	191	198	228
Diol–ethanol ratio	4.4	4.5	8.6
C recovery, %	103.9	102.5	109.4
Fermentation time, hr.	21	24	72
Sugar used, %	99.3	98.8	70.7
Initial pH (32°C.)	5.65	6.83	6.62
Final pH (32°C.)	5.49	5.87	5.15

TABLE V

AEROBIC DISSIMILATION OF 5% SUGAR IN CHATHAM 1946 MOLASSES BY *Aerobacter aerogenes*

(mM. per 100 mM. invert sugar fermented)

Product \ Strain	B199				Average B199	NRC M148
2,3-Butanediol + acetoin	74.2	84.8	85.5	87.3	82.9	84.5
2,3-Butanediol	55.7	78.7	73.3	66.3	68.5	79.2
Acetoin	18.5	6.1	12.2	21.0	14.4	5.3
Ethanol	40.1	42.7	41.0	34.5	39.6	44.5
Acids	8.0	8.6	10.3	....	9.0	....
Diol–ethanol ratio	3.6	3.9	4.1	4.9	4.1	3.7
C recovery, %	98.8	106.9	107.4	106.0	104.8	103.7
Fermentation time, hr.	24	24	24	26	24.5	21
Sugar used, %	99.2	100.0	99.3	99.3	99.4	99.3
Initial pH (32°C.)	5.40	5.40	5.40	5.65	5.46	5.65
Final pH (32°C.)	5.43	5.46	5.45	6.21	5.74	5.28

from acetone–water. Polarization of 0.5 gm. of the dry mixed esters in 25 ml. chloroform gave $\alpha_D^{25} = -0.029$. The percentage of *levo*-diol is therefore approximately 1.5%. In three successive crops of crystals only two types were found:

(1) large, transparent, light yellow; forming rosette-like clusters; m.p. 128° – 132°C. (uncorr.). These crystals were therefore *dl*.

(2) fine, opaque, almost colorless; m.p. 192° – 194°C. (uncorr.). These crystals were therefore *meso*. The proportions of *meso*- and *dl*-crystals were approximately equal.

Discussion

Variations in initial pH from 5.0 to 5.8 cause only minor variations in fermentation time and product yield (Table I). This fact suggests that *P. hydrophila* 492 may be less affected by increasing hydrogen-ion concen-

tration than other 2,3-butanediol producing bacteria (8, 21). The effect of varying pH on glucose dissimilation by Reed's R2 strain (N.R.C. 492) shows an increasing yield of diol + acetoin with decreasing pH from 6.7 to 4.0 (18). A maximum yield (85.4%) of diol + acetoin is obtained from 5% glucose in 44 hr. at pH 4.0. At pH 3.8 the yield of organic acids is so low (1.5 mM. per 100 mM. of glucose fermented) that even in a slightly buffered synthetic medium the acids formed during the fermentation do not suffice to hold the pH below 4.0. At pH 4.2 the fermentation of 5% glucose is complete in 25 hr. with a yield of 82.3% diol + acetoin. Maximum yields of diol from glucose therefore occur near the lowest pH level attained in slightly buffered media. The natural buffers present in beet molasses hold the pH above 5.0 (see results in Table I).

Freeman and Morrison (3) investigated the rate of sucrose conversion and 2,3-butanediol yield by a number of strains of *A. aerogenes* from different sources in England and America. None proved superior to NRRL strain B199. The data in Tables I and V indicate that *A. aerogenes* B199 completes the fermentation of 5% molasses sucrose in approximately the same time as *P. hydrophila* 492, but that the latter organism produces a higher yield of glycol + acetoin. Aeration has less tendency to convert diol to acetoin in the *P. hydrophila* fermentation. The higher yields of glycol from *Pseudomonas* are correlated with lower yields of ethanol, although the factors controlling ethanol formation by this organism have not been investigated (10).

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THE OXIDATION, DECOMPOSITION, IGNITION, AND DETONATION OF FUEL VAPORS AND GASES

XVIII. THE OPERATION OF A CARBURETOR TYPE ENGINE BY COMPRESSION IGNITION WITH DIETHYL ETHER OR ACETALDEHYDE AS THE FUEL[1]

By R. O. King,[2] E. J. Durand,[3] and A. B. Allan[4]

Abstract

Diethyl ether and acetaldehyde knock violently when used as fuels for carburetor engines in normal temperature and mixture strength conditions. The experiments described in this Part show however that in relatively low temperature conditions both substances can be used at any ignitable mixture strength at compression ratios varied accordingly and that, in the circumstances, ignition can be either by spark or compression. The nuclei required for ignition by compression are believed to be the fog particles which are known to be formed in heated mixtures of air with ether or acetaldehyde prior to explosive ignition.

Introduction

Diethyl ether and acetaldehyde are, without qualification, stated in works of reference to be fuels of extremely low anti-knock value. Thus, Pye (8, p. 85), states that the ether detonates more readily than any other known substance. Egerton, Smith, and Ubbelohde (2, p. 464) report being unable to use acetaldehyde as the fuel for an ethyl knock testing engine because of the violence of knocking combustion.

It was shown, however, by experiments described in Part X (5) that acetaldehyde could be used as the fuel for a C.F.R. knock testing engine, in conditions of an unheated air supply and a relatively low jacket temperature. Ignition was by spark with advance fixed at 10°. The experiments described in this Part show that in similar temperature conditions diethyl ether can also be used as the fuel and that compression ignition can replace spark ignition for either of the fuels.

Experimental Conditions and Methods

Temperature and Humidity of Air Supply

Relatively dry air is required to avoid ice formation in the carburetor when using low boiling point fuels. Suitable air conditioning equipment not being available, it was necessary to carry out the experiments in mid-winter, with the engine drawing the air supply from outside the laboratory. Weather conditions were unfavorable during the greater part of the period, 16 Dec. 1949

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Contribution from Defence Research Board, Ottawa, Canada, in association with the Department of Mechanical Engineering, University of Toronto, Toronto, Canada.
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[4] *Research Assistant, Mechanical Engineering, University of Toronto.*

to 7 Jan. 1950, during which the C.F.R. engine was available. Even when it was possible to run without ice forming in the carburetor, temperature and humidity varied during the time required for a comprehensive trial. Every measurement of power and air to fuel ratio was corrected accordingly.

Vapor Lock in Fuel Supply System

Special precautions are necessary to prevent vapor lock in the fuel system when using low boiling point fuels. The effect was avoided by shielding the carburetor bowls and the fuel metering vessels from heat radiation from the engine and by cooling the engine cubicle.

Engine Speed and Charge Density

The engine speed was maintained at 400 r.p.m. by adjustment of load, for all of the experiments. The air supply was unrestricted, the carburetor being bolted directly to the engine head and the standard shrouded inlet valve replaced by one of the ordinary variety. An exceptionally high charge density for a C.F.R. engine was thus obtained, especially in the low temperature conditions of the experiments.

Standard Knock Intensity

The bouncing pin contacts were so adjusted that a midscale knockmeter reading was obtained for a standard degree of audible knock intensity when using pentane as the fuel. The intensity was moderately heavy. The standard intensity of knock was then taken as having been obtained for ether or acetaldehyde on adjusting the compression ratio for a midscale knockmeter reading.

Ignition Arrangements

Spark ignition timing was set arbitrarily at 10° advance and was not adjusted to an optimum value as mixture strength or compression ratio was varied and the engine ran at a low compression ratio without knocking combustion. The engine would, however, run apparently indefinitely without spark ignition on raising the compression ratio to obtain standard knock intensity.

Experimental Results

Diethyl Ether

The ether $(C_2H_5)_2O$ boils at 34.6°C. (94.3°F.), at normal atmospheric pressure. The heat of vaporization is 151.0 B.T.U. per lb. The lower calorific value of 14,567 B.T.U. per lb. was used for calculations of thermal efficiencies. The "correct" air to ether ratio by weight is 11.2: 1.

Temperature Conditions

Air temperatures outside and inside the laboratory differed little during the experiments. The inside temperature was 47.5°F. at the beginning of the experiments and rose to 50°F. while they were in progress. The outside temperature fell from 46.1°F. to 45.3°F. during the same period. Thus, as

the outside air was drawn by the engine through 20 ft. of 3 in. bare steel pipe inside the laboratory, a compensating effect occurred and the air temperature at the carburetor inlet remained nearly constant at 50°F. The relative humidity of the outside air diminished from 63 to 55% during the experimental period and there was no formation of ice in the carburetor. The temperature of the ether – air mixture entering the cylinder varied from 30.5° to 7.8°F., depending on the rate of supply of ether. Jacket cooling was by an automatic thermostatic control of a water circulation. The temperature of the water was 75°F. at the entrance to the cylinder jacket and 80°F. at the exit from the engine head jacket.

Compression Ratio for Standard Knock Intensity and Corresponding Values of Power and Thermal Efficiency

The values obtained as a result of experiments made over the widest possible range of mixture strength are given by the graphs of Figs. 1 and 2. The compression ratio for standard knock intensity, as indicated by a midscale knockmeter reading, will be described as the "usable compression ratio" and abbreviated to U.C.R. Fig. 1 exhibits values of U.C.R., and corresponding values of brake and indicated horsepower and of brake and indicated thermal

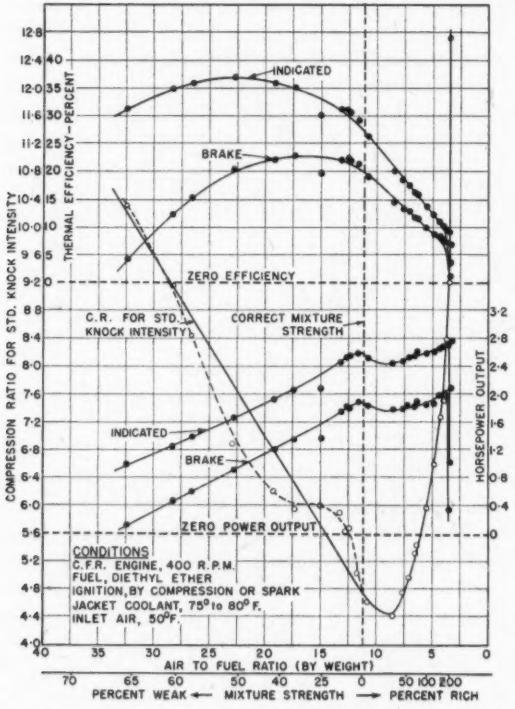

FIG. 1. *Diethyl ether, experimental results plotted on a linear base of air to fuel ratio.*

efficiency plotted on a linear scale of ether–air ratios by weight and the corresponding mixture strengths in percentages weak or rich as given by a nonlinear scale. The method of plotting expands the graphs on the weak side of the correct mixture strength. They are expanded on the rich side, Fig. 2, by plotting values on a linear scale of rate of ether consumption with corresponding mixture strengths given as percentages of the correct value.

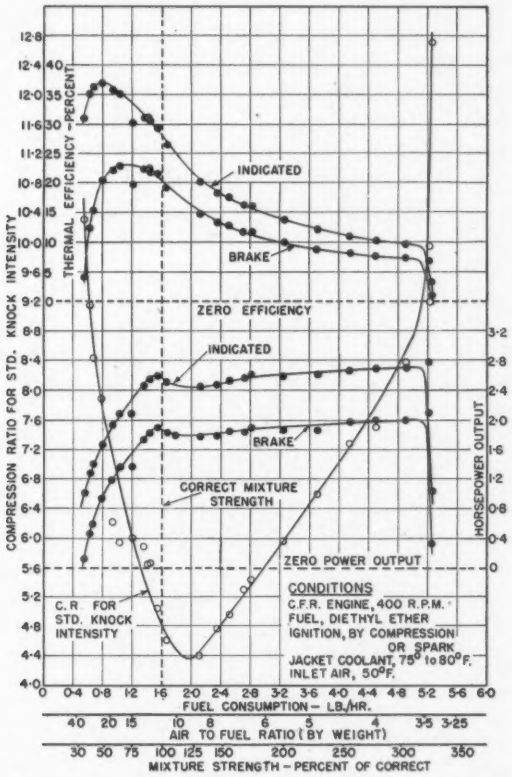

FIG. 2. *Diethyl ether, experimental results plotted on a linear base of rate of fuel consumption.*

It will be noted that the experimental observations for the U.C.R. of mixtures 10 to 60% weak do not fall on the graph as drawn. The observations indicate that the graph might be drawn as shown by the broken line, Fig. 1. The inflection obtained accordingly was attributed, at first, to experimental error but frequent occurrence in subsequent experiments indicates that it was due to a real effect; the cause being unknown at present.

It was observed in the course of the experiments that at extremely rich or weak mixtures, the audibility of knocking combustion did not correspond with knockmeter readings although standard knock intensity was indicated.

Thus knock was barely audible at a mixture 66% weak, U.C.R. being 10.3: 1 but standard knock intensity was indicated by the knockmeter. A significant feature was that audible knock could be obtained by advancing the spark or by switching it off and raising the compression ratio. That is, when running on compression ignition, ignition timing was set by compression ratio. Somewhat similar effects were obtained with extremely rich mixtures but were always accompanied by heavy and irregular audible knock.

The engine ran steadily on compression ignition at intermediate mixture strengths; the audibility of knocking combustion at the U.C.R. corresponding approximately to the knockmeter reading. The U.C.R. for the correct mixture was 4.8: 1 and diminished to a minimum of 4.4: 1 for a mixture 20% rich; the corresponding values of indicated thermal efficiency being $27\frac{1}{2}$ and $21\frac{1}{2}$% respectively.

Indicated thermal efficiency attained a maximum value of 37% for a mixture 50% weak, U.C.R. being 7.6: 1. The value diminished as the mixture was further weakened and the compression ratio raised to maintain a midscale reading of the knockmeter. At a mixture 66% weak, brake horsepower was nearly zero, the U.C.R. 10.4: 1 and the indicated thermal efficiency 31%. It appeared that still weaker mixtures could have been used if power had been supplied to compensate, in part, for that lost in overcoming engine friction.

Engine performance on the rich side of correct mixture strength was somewhat remarkable. It is exhibited especially clearly by the graphs of Fig. 2. It will be noted that the engine continued to run and that power, after passing through a minimum value, continued to increase with increase of mixture strength and compression ratio until it dropped suddenly to nearly zero at a mixture just over 200% rich. A similar drop in power which occurred in later experiments was found to be due to loss of compression pressure caused by rotation of worn piston rings until by an odd chance the gaps provided a nearly straight through passageway from the combustion chamber to the crank case.

Acetaldehyde

The boiling point of acetaldehyde, CH_3CHO, is 21°C. (69.8°F.) at normal atmospheric pressure. The heat of vaporization is 244.8 B.T.U. per lb. The lower calorific value of 10,550 B.T.U. per lb. was used for calculations of thermal efficiencies. The "correct" air to acetaldehyde ratio by weight is 7.84: 1.

Temperature Conditions

The experiments were carried out during a period of falling temperature and rising humidity. The laboratory temperature was 50.5°F. at the beginning of the period and 45.5°F. at the end. The outside temperature was 42°F. at the beginning of the experiments and 36°F. at the conclusion. The relative humidity increased from 42% to 54% during the experimental period but there was no formation of ice in the carburetor. The temperature of the air

entering the carburetor diminished from 54°F. to 47° during the experiment and the temperature of the air–fuel mixture entering the cylinder varied from −7.0° to −8.5°F., depending on the rate of acetaldehyde supply. Water was supplied to the cylinder jacket at a temperature of 85°F. The temperature at the outlet from the engine head jacket was 90°F.

Compression Ratio for Standard Knock Intensity and Corresponding Values of Power and Thermal Efficiency

The experiments were carried out in the manner described for those made with ether as the fuel. The results are exhibited by the graphs of Fig. 3 plotted on a linear scale of rate of acetaldehyde consumption with corresponding mixture strengths given as percentages weak or rich. The acetaldehyde was, in the experimental conditions, usable over a wide range of mixture strengths on the rich side of the correct value but unlike ether the usable range was narrow on the weak side, extending to mixtures no weaker than approximately 25%.

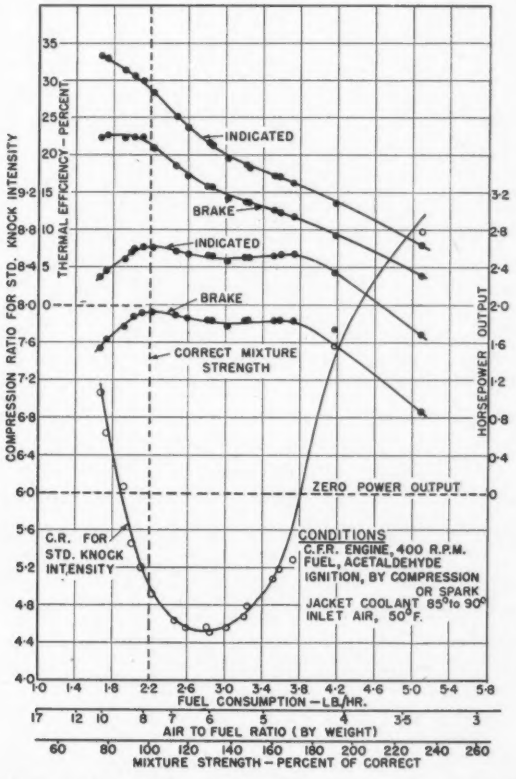

FIG. 3. *Acetaldehyde, experimental results plotted on a linear base of rate of fuel consumption.*

It was possible to run the engine on compression ignition at any mixture strength within the range usable with spark ignition if the compression ratio were sufficiently high to be accompanied by audible knock. The running on compression ignition was without irregularity over the mixture range from 10% weak to 170% rich and the audibility of knocking combustion could be varied over a wide range by varying the compression ratio. Running became irregular as the weak or rich mixture limit was approached. Compression ignition of mixtures near the rich limit was accompanied by irregular heavy knock.

Discussion

When a carburetor engine continues to run with spark ignition switched off, the necessary fuel igniting effect is commonly attributed to the required high temperature having been attained by a surface in the combustion chamber. The igniting effect, usually described as preignition, is most commonly observed when a hot engine has been operated at maximum power. Thus two means only of ignition are recognized as applying to the carburetor type engine, namely, spark ignition and ignition by overheated surfaces.

It was shown by experiments described in Part X (5) that preignition generally followed the partial oxidation of the fuel to aldehydes, substances which are commonly believed to be ignitable at lower temperatures than the hydrocarbons from which they are formed by partial oxidation. Thus compression ignition of *n*-pentane in the C.F.R. engine occurred at a compression ratio of 6.9: 1, Part XVII (6), whereas in the experiments of this Part made in similar conditions of engine temperature and mixture strength, a compression ratio of 4.5: 1 only was required to ignite acetaldehyde. It appears, therefore, that early compression ignition, as well as early surface ignition, may be due to aldehydes formed by partial oxidation of hydrocarbon fuels.

The nuclear theory of ignition as stated in Part IV (4) was advanced to account for the autoignition and consequent detonation of the unburned combustible mixture ahead of the flame front in a carburetor engine using hydrocarbon fuels, the nuclei of ignition being finely divided carbon derived from the cracking of the hydrocarbon at the high temperatures and pressures then attained. It was shown by the experiments described in Part XVII (6) that autoignition of the mixture could occur in suitable conditions at the lower temperatures and pressures attained before initiation of flame by a spark; that is, before there was any end gas. The igniting effect was obtained on running the engine at the relatively low speed of 400 r.p.m. and on admitting the fuel, in part, as liquid drops. That is, the conditions were suitable for the liquid phase cracking of the fuel to yield an igniting concentration of finely divided carbon in the time available. The layer of nonadherent carbon deposited on the piston crown in the course of the experiments afforded ample evidence that cracking had occurred.

Tizard and Pye (10) mention that carbon was "thrown down" when ether–air mixtures were suddenly compressed in their machine, to an ignition

temperature of 227°C (440°F). A similar effect was obtained on compressing pentane–air mixtures to an ignition temperature of 300°C (572°F). The temperatures mentioned are minimum values as calculated by Newitt and Townend (7, p. 2962).

The engine experiments of this Part were begun with nearly clean surfaces in the combustion chamber. A deposit of finely divided carbon was visible on water cooled surfaces after rich mixtures containing either acetaldehyde or ether had been used but not after using weak mixtures. If the autoignition of such mixtures be regarded as a nuclear effect, it is necessary to assume that the nuclei were consumed or carried out with the exhaust.

Callendar, when formulating a nuclear theory of ignition (1, p. 21), mentioned co-aggregated molecules as possible nuclei of ignition and initiated appropriate experiments which were carried out by Gill, Mardles, and Tett (3). It was shown by their experiments that explosive ignition of ethyl ether and acetaldehyde in mixtures with air was always preceded by the formation of fog. Ether oxidizes readily to aldehyde and the fog particles, which were so small that an intense beam of light was required to disclose their existence, may be regarded as molecular aggregates of condensation products of aldehyde.

The formation of fog occurs at relatively low temperatures when mixtures of air with ethyl ether or acetaldehyde are heated. Thus it is necessary, if the substances are to be used as engine fuel without excessive knocking combustion, to ensure that mixture temperature at the beginning of compression is exceptionally low. An igniting concentration of nuclei may otherwise be obtained at compression temperatures attained before the completion of the compression stroke, even when the compression ratio is as low as 4:1. An explanation is obtained accordingly for the inability of Ricardo to run the E 35 engine with ethyl ether as the fuel (9, p. 249) in normal operating conditions and for the failure of Egerton, Smith, and Ubbelohde to run the ethyl knock testing engine in similar conditions when using acetaldehyde as the fuel (2, p. 464).

Acknowledgments

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MICROBIOLOGICAL FORMATION OF VITAMIN B_{12}

I. PRODUCTION IN FISH PRESS LIQUID[1]

By H. L. A. Tarr

Abstract

Vitamin B_{12a} was formed by *Streptomyces griseus* species and by *Streptomyces aureofaciens* in aerated herring press water containing 2 μgm. per ml. of added cobalt (as cobaltous nitrate). The yield of the vitamin was improved by diluting the press liquid (stickwater) to 2% solids content. No consistent agreement was obtained in vitamin B_{12} assays of the crude fermentation liquors by two very different procedures. Highest apparent recovery of vitamin B_{12a} in these products (about 1100 mμgm. per ml. with *S. aureofaciens* and 800 mμgm. per ml. with *S. griseus*) was obtained by chromatography on potassium dihydrogen phosphate-treated filter paper strips, elution of the vitamin, and aseptic addition of the eluates to previously sterilized *Lactobacillus leichmannii* assay medium. Treatment of the crude fermentation products with potassium cyanide (2.5 mgm. per ml.) caused partial resolution of the vitamin B_{12a} to vitamin B_{12}.

Introduction

Though vitamin B_{12} was first obtained from liver (6, 18, 23) its isolation from *Streptomyces griseus* cultures was announced shortly afterward (19). However, very little information has as yet been made available regarding the media used, fermentation conditions, or yield of vitamin B_{12} by *Streptomyces* species. Hendlin and Ruger (10), in studying the influence of cobalt on vitamin B_{12} formation, recorded with *S. griseus* a yield of 3500 LLD units (about 320 mμgm.) of vitamin B_{12} active substances per ml. of a medium containing enzymic casein digest, beef extract, and about 2 μgm. per ml. of cobaltous ions. The role of cobalt in improving the yield of vitamin B_{12} in an aerated medium containing 10% sucrose and inoculated with *Bacillus megatherium* was recognized independently by Lewis and associates (8, 14). These investigators reported maximum yields of 800 mμgm. of alkali labile vitamin B_{12}-active substances per ml. of medium. The formation of vitamin B_{12}-active substances by various bacteria has been reported (10, 25), though the amounts formed appear to have been usually considerably lower than those recorded by the above workers. Very small yields of vitamin B_{12} were reported in nonaerated cultures of microorganisms isolated from poultry house litter (9).

Recent work has shown that several forms of vitamin B_{12} may occur (1), and this property has been attributed to the ability of the cobalt moiety to form co-ordination complexes with different ions (5). Vitamin B_{12} is regarded as a cyano cobalt co-ordination complex (11) or "cyano cobalamine", and vitamin B_{12a} as the corresponding hydroxy complex (5). It is now generally accepted that *Streptomyces* species form largely vitamin B_{12a} (7, 15), and that this form is identical with vitamin B_{12b} (1, 11). Vitamin B_{12a} has been found

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to yield much lower results in an assay procedure using *Lactobacillus leichmannii* than in a rather similar procedure using *L. lactis* (27). Thus it has been stated recently that autoclaving vitamin B_{12a}, in an *L. lactis* assay medium which contains no added reducing agent such as ascorbic acid, occasions about 30% destruction of the vitamin (11). It appears probable that the almost universal employment of reducing agents in *L. leichmannii* assay media may be responsible for the extensive destruction of vitamin B_{12a} which occurs during autoclaving (27). This contention is supported by the report that vitamin B_{12a} is much less stable in presence of ascorbic acid than is vitamin B_{12} (26), though vitamin B_{12} is itself unstable in the presence of ascorbic acid and other reducing agents if the concentration is fairly high (13).

The present work was undertaken with the object of determining the feasibility of enhancing, by microbiological fermentation, the natural, rather variable, vitamin B_{12} content of fish materials (17, 25). The results obtained in preliminary experiments have been described previously (24, 25).

Cultures, Materials, and Methods

S. aureofaciens and five strains of *S. griseus* obtained from different laboratories were used, and were maintained by weekly transfer on Bacto nutrient agar followed by room temperature incubation. For microbiological assay of vitamin B_{12} *Lactobacillus leichmannii* (A.T.C.C. No. 4797) and a vitamin B_{12}-requiring *Escherichia coli* mutant (British N.C.I.B. No. 8134) were employed.

Herring press liquid (October 1948) was frozen and stored at −20°C. to −28°C. until required for the fermentation experiments. This material is known to be a fair source of essential amino acids (16). The particular sample used contained 7.8% total solids, 0.95% ash, 0.85% sodium chloride, and its pH was 6.8. In one experiment a tryptic casein digest–beef extract medium (10) was used (pH 6.8), the tryptic digest being prepared by the method of Kitay, McNutt, and Snell (12), except that it was not adsorbed with charcoal.

Fermentation of the press liquid with *Streptomyces* species was carried out as follows. The material, containing 2 μgm. per ml. of added cobaltous ions (10), was sterilized for 15 min. at 120°C. either in 25-ml. quantities in 3 × 20 cm. test tubes, or in 150-ml. quantities in 7 × 28 cm. Pyrex wash bottles. The containers were closed with rubber stoppers which were equipped with the conventional glass inlet and exhaust tubes for aseptic aeration of the medium. In the smaller containers, glass tubes with constricted tip were used for aeration, while in the larger containers, coarse sintered-glass Pyrex discs were employed for this purpose. The press liquid was inoculated with a small piece of the growth from a two-day nutrient agar culture of the *Streptomyces* species being studied, and it was then aerated vigorously at 28°C. with air sterilized by passage through dilute sulphuric acid, dilute sodium hydroxide,

sterile absorbent cotton, and sterile distilled water. Serious foaming rarely occurred, and in the occasional instances where it did occur, it was counteracted successfully by addition of a few drops of sterile herring oil. Small samples of culture liquid were removed at intervals for vitamin B_{12} assay, and the volume was kept constant by addition of sterile distilled water.

Two methods of vitamin B_{12} assay were used. In the first, the tube assay technique described by Skeggs, Nepple, Valentik, Huff, and Wright (22) was followed with the following minor modifications. The double strength medium was adjusted to pH 6.9, and *l*-ascorbic acid (1 mgm. per ml.) was added immediately before automatically dispensing 2-ml. quantities in 18 × 150 mm. tubes, the final pH being 6.5. In most experiments, vitamin B_{12} solution (0.2 mμgm. per ml.), or appropriately diluted fermentation product, was added, together with distilled water, to bring the total volume to 4 ml. prior to sterilizing for five minutes at 120°C. However, when the advantage of adding vitamin B_{12a}-containing materials aseptically to the previously sterilized medium was realized (*vide infra*), this modification was employed. In order to facilitate the rapid uniform growth essential for satisfactory turbidimetric assays it was found advantageous to inoculate each tube with 0.1 ml. of a 1: 100 water dilution of a 16-hr. culture of *L. leichmannii* grown in the assay medium containing 0.1 mμgm. per ml. of vitamin B_{12}. The vitamin B_{12}-containing broth cultures used for inoculation were prepared from stock cultures each week, transferred daily, incubated for 16-18 hr., and kept at 0°C. until subsequent transfers were made. *L. leichmannii* assays were incubated at 37°C. for 18 hr., the amount of growth being determined turbidimetrically using a Beckman model DU spectrophotometer set at 540 mμ and 0.1 mm. slit width. Tests showed that approximately 20,000 times as much thymine or guanine deoxypentoside as vitamin B_{12} was required to give similar growth response in this assay.

The second assay method employed was a cup–plate procedure using a simple inorganic salt–glucose medium solidified with 2% agar and inoculated with the *E. coli* mutant (2, 3). With this method the diameter of the growth-exhibition zones varied directly with the logarithm of the concentration of vitamin B_{12} when concentrations of the order of from 1 to 1000 mμgm. per ml. were placed in the assay cylinders. Fermentation products were adjusted to pH 6.8 and heated 30 min. at 100°C. before being assayed by this method.

Separation of vitamin B_{12} and B_{12a} in fermentation products was effected by chromatography on potassium dihydrogen phosphate treated Whatman No. 1 filter paper strips with water-saturated *n*-butanol as solvent (27). Experience showed that for quantitative recovery, it was advisable to apply 0.02 to 0.10 ml. of the fermented liquid to the strip, the larger volumes being spotted by several successive applications of 0.01 or 0.02 ml. An Agla micrometer syringe proved useful for these applications, particularly since fermentation products containing rather large mycelial "pellets" could be "emulsified" by repeated passage through the glass nozzle. All operations were

carried out either in the dark or in subdued light. Duplicate strips were always prepared, and the position of the vitamin B_{12} and vitamin B_{12a} was located by "developing" one strip on the inorganic salt – glucose agar medium inoculated with the *E. coli* mutant. With this method, growth zones corresponding to vitamin B_{12} or B_{12a} developed in about six hours at 37°C., but, for convenience, an overnight incubation temperature of 28°C. was generally used. Quantitative vitamin B_{12} or B_{12a} determinations were carried out using aqueous solutions obtained by eluting corresponding areas on the duplicate filter paper strips into sterile distilled water in experiments in which aseptic additions were made to the assay medium.

Thymine deoxyriboside (m.p. 186° – 187°C.; Micro block) was made by the method of Brady (4) and guanine deoxyriboside by that of Schindler (21).

Experimental

In order to test the reliability of the *L. leichmannii* vitamin B_{12} assay method, five determinations were carried out at different times on herring press water with the following results: 97, 97, 102, 103, and 105 mμgm. per ml. (average 101). This experiment indicated that the results obtained with this crude material were reproducible within limits of ± 4%. A similar degree of accuracy can normally be obtained with aqueous extracts of fish or mammalian tissues or organs. However, as will appear later, the recovery of vitamin B_{12} in fermentation products depends to an important extent on factors other than the accuracy of the assay technique itself. The same material, when assayed by the *E. coli* cup–plate method, had an apparent vitamin B_{12} concentration of 75 mμgm. per ml. Vitamin B_{12} formation by all six *Streptomyces* species was studied in a number of preliminary experiments using 25-ml. portions of undiluted press liquid. There was no very pronounced difference in the amount of vitamin B_{12} produced by the various organisms under the experimental conditions, therefore only two of the *S. griseus* cultures, and *S. aureofaciens*, were subjected to more detailed study.

The results obtained in four experiments using undiluted herring press liquid are recorded in Table I. There was an apparent decrease in the vitamin B_{12} content of the aerated sterile press liquid (controls), and whether this was due to actual destruction or change to a form (e.g. vitamin B_{12a}) which yielded lower results in the normal assay procedures with *L. leichmannii* (*vide infra*) was not determined. With all *Streptomyces* species there was a definite but limited increase in the vitamin B_{12} content. In these, and in subsequent experiments, antibiotic produced by *S. aureofaciens* usually markedly suppressed growth in *L. leichmannii* assays.

In a subsequent experiment the yield of vitamin B_{12} in herring press liquid diluted to 2% solids content, and in casein digest – beef extract medium, was determined by the two different assay techniques. The susceptibility of the vitamin B_{12}-active substances, formed in these media, to destruction when

heated for 30 min. at 100°C. in 0.2*N* sodium hydroxide, was also determined in some instances. The results of a typical experiment are given in Table II. There was a marked increase in the vitamin B_{12} content of both substrates, especially after four days' fermentation, the increase being most marked with

TABLE I

VITAMIN B_{12} CONTENT OF UNDILUTED HERRING PRESS LIQUID AFTER FERMENTATION WITH *Streptomyces* SPECIES

Experiment No.	Period of fermentation (days)	Vitamin B_{12} (mμgm. per ml.)			
		Control (uninoculated)	*S. griseus* A.T.C.C. 10137	*S. griseus* 3478	*S. aureofaciens*
1	4	55	220	180	260
2*	2	50	140	160	210
3	2	75	100	...	...
	3	50	190	160	**
4	2	50	120	150	**

**Inoculated with fermented material from the corresponding sample in Expt. 1.*
***Antibiotic present prevented tube assays from being carried out successfully.*

TABLE II

VITAMIN B_{12} CONTENT OF HERRING PRESS LIQUID DILUTED TO 2% SOLIDS CONTENT, AND OF A CASEIN DIGEST – BEEF EXTRACT MEDIUM, AS DETERMINED BY TWO DIFFERENT MICROBIOLOGICAL ASSAY PROCEDURES CARRIED OUT ON FERMENTED LIQUIDS

	Vitamin B_{12} (mμgm. per ml.)			
	Fermented 2 days		Fermented 4 days*	
	L. leichmannii assay	*E. coli* assay	*L. leichmannii* assay	*E. coli* assay
Herring press liquid				
Uninoculated	1.8	2.0	3.4	12.0
Inoculated with:				
S. griseus 10137	240	150	480(0.6)	750(0)
S. griseus 3478	90	55	250(0.4)	750(0)
S. aureofaciens	...	150	250(0.2)	1300(0)
Casein digest – beef extract medium				
Uninoculated	0.05	...	0.2	...
Inoculated with:				
S. griseus 10137	180	170	200(2.2)	750(2.5)
S. griseus 3478	60	40	100(3.0)	600(2.0)
S. aureofaciens	...	About 5	...	180

** The bracketed figures are the results obtained after heating the samples in 0.2 N sodium hydroxide for 30 min. at 100°C.*

S. griseus A.T.C.C. No. 10137 and *S. aureofaciens*. In general, the yield of vitamin B_{12} was higher in the press liquid than in the casein digest – beef extract medium. There was no agreement between results of assays by the tube and the cup–plate methods, though the results obtained by the latter method were invariably considerably higher after four-day fermentation. Treatment with alkali under conditions which are known to lead to destruction of vitamin B_{12} caused complete, or almost complete, elimination of vitamin B_{12}-active substances. Difficulty was again experienced in obtaining satisfactory vitamin B_{12} assays with the *S. aureofaciens* products, particularly in the casein digest medium.

A further experiment was made in which 150-ml. quantities of press liquid, adjusted to 2% solids content, were fermented, and samples were withdrawn at intervals for microbiological assay for vitamin B_{12} by the two different methods. The results (Table III) show a definite increase in vitamin B_{12} in

TABLE III

VITAMIN B_{12} CONTENT OF HERRING PRESS LIQUID DILUTED TO 2% SOLIDS CONTENT AS DETERMINED BY TWO DIFFERENT MICROBIOLOGICAL ASSAY PROCEDURES CARRIED OUT ON THE FERMENTED LIQUORS

Days fermented	Vitamin B_{12} (mμgm. per ml.)					
	S. griseus No. 10137		*S. griseus* No. 3478		*S. aureofaciens*	
	L. leichmannii assay	*E. coli* assay	*L. leichmannii* assay	*E. coli* assay	*L. leichmannii* assay	*E. coli* assay
2	110	380	70	50	20	330
3	320	500	110	110	100	100
4	460	250	150	170	310*	120
5	390	180	190	170	220*	50

* *Strong growth suppression in the lower dilutions rendered accurate assays impossible.*

the fermented press liquid, especially with *S. griseus* A.T.C.C. No. 10137 and *S. aureofaciens*. Again there was no agreement between the two vitamin B_{12} assay methods except with the *S. griseus* No. 3478 fermentation product. With the other two products there was a tendency for the *L. leichmannii* assay to indicate a steady increase in vitamin B_{12} up to the fourth day, while with the *E. coli* method there was an apparent decrease in vitamin B_{12} during the latter part of the fermentation. These findings, when taken in conjunction with those reported in the foregoing experiments, indicate that direct assay of crude fermentation products for vitamin B_{12} may not yield very reliable results.

In view of the variable results obtained in the direct assay of the crude fermentation products for vitamin B_{12}, concentration and partial purification of the vitamin by chromatographic separation on filter paper strips, followed by elution and microbiological assay of the eluates, was carried out. Since

previous work (25) had indicated that chromatography using ordinary untreated filter paper strips cannot always be relied upon to separate vitamin B_{12} from interfering vitamin B_{12}-active substances, the method of Woodruff and Foster (27), by which vitamin B_{12} and B_{12a} are separated not only from interfering substances but also from each other, was adopted. Since vitamin B_{12} is unstable toward light (6) all chromatographic procedures were carried out either in the dark or at least in subdued light.

Preliminary trials with crystalline vitamin B_{12} verified the fact that only about 90% of the vitamin B_{12} chromatographed is recovered as such, and that the remainder appears as vitamin B_{12a} (27). The results given in Table IV

TABLE IV

RECOVERY OF VITAMIN B_{12} AND B_{12a} USING CHROMATOGRAPHY OF CRYSTALLINE VITAMIN B_{12} ON KH_2PO_4 TREATED FILTER PAPER, FOLLOWED BY ELUTION AND *L. leichmannii* ASSAY OF THE AQUEOUS ELUATES

Vitamin B_{12} applied (mμgm.)	Vitamin B_{12}, mμgm.		Vitamin B_{12a}, mμgm.	
	Expected*	Recovered	Expected**	Recovered
150	135	133	15	1.8
"	"	139	"	6.2
75	67.5	62	7.5	0.44
"	"	65.3	"	1.24
15	13.5	13.6	1.5	0.46
"	"	13.9	"	0.56
7.5	6.75	6.8	0.75	0.36
"		6.51	....	....
1.5	1.35	1.60	....	....
"		1.49		

* *Assuming 90% of the vitamin B_{12} applied appears as such.*
** *Assuming 10% " " " B_{12} " " " vitamin B_{12a}.*

show that, within reasonable limits of error, it was possible to recover almost quantitatively all the vitamin B_{12} when amounts of from 1.5 to 150 mμgm. were applied to the paper strips. Very poor and variable recovery of the expected vitamin B_{12a} was experienced, and in view of later work (*vide infra*), it would appear that autoclaving of this form of the vitamin in assay medium containing ascorbic acid, was responsible for the loss.

It was found that separation of the vitamin B_{12} and B_{12a} in press liquid fermentation products could be effected by spotting 0.01 to 0.10 ml. on potassium dihydrogen phosphate treated filter paper strips and developing these for 48–52 hr. at 37°C. In these products, especially after prolonged fermentation, all, or nearly all, of the vitamin occurs as vitamin B_{12a}.

All subsequent quantitative studies were carried out using the five-day *S. aureofaciens* and *S. griseus* A.T.C.C. 10137 products from the foregoing experiment, these being stored under benzene at 0°C. The *S. griseus* product was rather viscous and considerable autolysis of the mycelia seemed to have

occurred, while the *S. aureofaciens* product contained masses of mycelial pellets. The *S. griseus* product was chromatographed in 0.02- or 0.10-ml. amounts, and the vitamin B_{12a} zone was eluted and assayed by *L. leichmannii* procedure. Values of 325, 355, 385, and 420 mμgm. per ml. were obtained in separate assays. The average value, namely 366, was very similar to that obtained in direct assay of the product.

At this stage, it became apparent that the *L. leichmannii* assay procedure would have to be modified in order to avoid extensive destruction of vitamin B_{12a}. Preliminary tests showed that aseptic addition of the appropriately diluted fermentation products, or eluates containing vitamin B_{12a}, to the separately sterilized assay medium gave considerably higher recovery than did their sterilization when included in the assay medium. A series of experiments was therefore carried out with the object of determining the optimum assay conditions for the determination of vitamin B_{12a} in fermentation products. In this work the following conditions were investigated: (1) aseptic addition of the unheated product, suitably diluted, to the previously sterilized assay medium, (2) identical with (1) only the product was adjusted to pH 6.8 and heated 30 min. at 100°C., (3) identical with (1) only the product was adjusted to pH 6.8 and heated 15 min. at 120°C., (4) the product was chromatographed, eluted, added to the assay medium, and sterilized as usual, and (5), identical with (4), only the eluates were added aseptically to the previously sterilized assay medium. The results of these experiments, which are recorded in Table V, show that the conditions under which microbiological assays of crude fermentation products are conducted have a very important bearing on the apparent recovery of vitamin B_{12}.

With the *S. griseus* product addition of the crude diluted material itself, or of aqueous eluates from filter paper chromatograms, to the assay medium before sterilization yielded rather similar assay values (385 to 420 mμgm. per ml.). Considerably higher values resulted when neutral aqueous solutions of the crude fermentation product were heated at 100° or 120°C. and were then added, after suitable dilution, to the separately sterilized assay medium. This procedure has the advantage that heating is believed to release "bound" forms of vitamin B_{12}(20), and that it avoids, to some extent at least, destruction of vitamin B_{12a}. When chromatographic separation of the vitamin B_{12a} followed by aseptic addition of the eluates to sterile assay medium was employed, even higher apparent recovery of the vitamin resulted. With the *S. aureofaciens* product results were somewhat confused by the fact that antibiotic produced made the direct assay of the crude material very difficult. However, with this product the highest apparent vitamin B_{12a} yield also resulted when chromatographic separation followed by aseptic addition of eluates to the sterile assay medium was employed. It is suggested that, at least until further information becomes available, this last named technique is more liable to yield reliable information concerning the vitamin B_{12a} content of fermentation products than are the other methods.

TABLE V

EFFECTS OF MICROBIOLOGICAL ASSAY CONDITIONS, AND CHROMATOGRAPHIC SEPARATION PRIOR TO ASSAY, ON THE APPARENT VITAMIN B_{12a} CONTENT OF FERMENTATION PRODUCTS

Treatment	Vitamin B_{12a} (mμgm. per ml.)	
	S. griseus product	*S. aureofaciens* product
Original values obtained by addition of a 1: 1000 dilution of the products to the medium prior to sterilization	390	220*
A 1: 2000 dilution of the unheated product added aseptically to the previously sterilized medium	400	560*
Product heated at pH 6.8 for 30 min. at 100°C. and added aseptically to the sterilized medium as a 1: 2000 dilution	590	490*
Product heated at pH 6.8 for 15 min. at 120°C. and added aseptically to the sterilized medium as a 1: 2000 dilution	650	530*
Product (0.02 ml.) chromatographed and the eluate added to the medium prior to the sterilization	420	850
Product (0.10 ml.) chromatographed and the eluate added to the medium prior to sterilization	385	830
Product (0.02 ml.) chromatographed and the eluate added aseptically to the previously sterilized medium	{740 880	{1020 1040
Product (0.10 ml.) chromatographed and the eluate added aseptically to the previously sterilized medium	830	{1040 1140

** These figures are not very accurate since antibiotic present prevented growth in assay tubes containing the lower dilutions.*

It has recently been reported that treatment of crystalline vitamin B_{12a} with cyanide at pH 5–6 causes conversion of this "hydroxy cobalamine" form to the "cyano cobalamine" form (vitamin B_{12}) (11). Treatment of the crude fermentation products at room temperature and pH 5.5 with about the same concentration of potassium cyanide (2.5 mgm. potassium cyanide per ml.) as used in the above work caused a partial conversion of the vitamin B_{12a} to vitamin B_{12} as indicated by quantitative chromatographic separation of the two forms on potassium dihydrogen phosphate treated filter paper strip, elution, and microbiological assay (Table VI). The results obtained in the

TABLE VI

PARTIAL RESOLUTION OF VITAMIN B_{12a} TO VITAMIN B_{12} IN FERMENTATION PRODUCTS TREATED WITH POTASSIUM CYANIDE

Cyanide treated product chromatographed	Vitamin B_{12} (mμgm. per ml.)	Vitamin B_{12a} (mμgm. per ml.)	Total (vitamin B_{12} + vitamin B_{12a})
S. griseus product, 0.10 ml.	198	175	373
" " , 0.10 ml.	168	208	376
" " , 0.01 ml.	100	260	360
S. aureofaciens product, 0.10 ml.	175	257	432
" " , 0.02 ml.	173	253	426

assay of the vitamin B_{12a} are probably low, since the experiments were carried out before it was realized that better recovery of this form of the vitamin could be obtained by aseptic addition of the eluates to the previously sterilized assay medium.

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